

EXHIBIT 20

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Stable Reduction of Thymidine Kinase Activity in Cells Expressing High Levels of Anti-Sense RNA

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Summary

Anti-sense thymidine kinase (TK) RNA was expressed as part of a chimeric dihydrofolate reductase (DHFR) anti-sense TK transcript. High level expression was obtained by selection of cells resistant to progressively higher levels of methotrexate, provided by overproduction of DHFR. The result was a concomitant increase in intracellular anti-sense TK RNA level owing to its presence on the same transcription unit as DHFR. In several cell lines expressing high levels of anti-sense TK RNA, thymidine kinase activity was reduced by 80%-90%. RNA:RNA duplexes were detected in the nuclear fraction. The results suggest a mechanism for diminution of TK activity; anti-sense RNA hybridizes with sense TK RNA in the nucleus, and duplex containing TK transcripts fail to enter the cytoplasm with normal efficiency.

Introduction

DNA sequence analysis provides detailed structural knowledge of cloned genes, but the functions of their protein products are in many cases incompletely understood, and in some instances entirely unknown. To analyze further the cellular function of a cloned gene, it is often informative to either specifically eliminate or block the function of the gene product of interest.

A method for establishing the null or hypomorphic phenotype, which is potentially general, utilizes anti-sense nucleic acids (Izant and Weintraub, 1984). In theory, sufficiently high intracellular concentrations of nucleic acids complementary to a given messenger RNA species will hybridize with that mRNA (for its precursor) in vivo, and such hybridization will ultimately inhibit synthesis of the protein product. There are a number of observations suggesting that this may be feasible. For example, in *E. coli*, expression of the IS 10 transposase gene (Simons and Kleckner, 1983) and the Omp F gene is apparently regulated at the translational level by anti-sense RNAs (Mizuno et al., 1984). Also, the activity of an RNA that serves to prime replication for the Col E1 plasmid appears to be reduced by annealing with a complementary RNA (Conrad and Campbell, 1979; Tomizawa et al., 1981). These results indicate that in several systems anti-sense RNAs exert regulatory effects by hybridizing with their complements in vivo. Nevertheless, it is important to recognize that proteins may play an important part in

mediating these hybridization events (e.g. see Tomizawa, 1984; Moser et al., 1984).

Several studies have investigated whether anti-sense nucleic acids can inhibit the expression of other gene products. In *E. coli*, expression of anti-sense IPP, Omp A, and Omp C RNAs from a lac Z promoter reduced the cellular concentration of the IPP, Omp A, and Omp C (Coleman et al., 1984), and lac Z proteins (Pestka et al., 1985). In frog oocytes, injection of anti-sense globin RNA into the cytoplasm inhibited synthesis of globin protein from microinjected beta globin messenger RNA (Melton, 1985). It was shown that RNA:RNA duplexes were formed in the oocyte after microinjection, suggesting that RNA hybridization was instrumental in establishing the observed block in globin protein synthesis. In *Drosophila*, microinjection of kruppel anti-sense RNA appears to cause a defect in embryo segmentation, which is a phenocopy of defects exhibited by kruppel mutants (Rosenberg et al., 1985).

Izant and Weintraub (1984) were the first to point out the potential of anti-sense RNA analysis and establish its feasibility in a model system. In mammalian cells, microinjection of thymidine kinase (TK) genes into TK negative cells typically results in a transient burst of TK synthesis. They discovered that if the recipient cells contain an excess of genes coding for anti-sense TK RNA, there is a large reduction in thymidine kinase expression from the injected genes. However, since microinjection experiments are confined to small cell numbers, direct investigation of the mechanism of enzyme depression was not possible. In addition, a further question becomes important if complementary RNA analysis is to be generally applicable; will anti-sense RNAs effectively reduce synthesis of gene products that are expressed from chromosomal genes, rather than transiently expressed from newly introduced DNA? These questions are addressed by the experiments reported here.

Results

Initial Anti-Sense Genes Fail to Direct Accumulation of Complementary RNA

In preliminary experiments, anti-sense dihydrofolate reductase (DHFR) or TK sequences were inserted between the metallothionein promoter and a metallothionein intron and polyadenylation addition site. The metallothionein gene was chosen because transcription can be induced by heavy metals, so that the expression of anti-sense transcripts could be regulated. Each of these recombinant genes was introduced into TK⁻ L cells by DNA mediated gene transfer. When these transformants were treated with heavy metals to induce transcription from the metallothionein promoter, little or no anti-sense RNA had accumulated as determined by RNA blotting analysis. Furthermore, neither TK nor DHFR enzyme levels were significantly reduced.

A major problem in these experiments was failure to ac-

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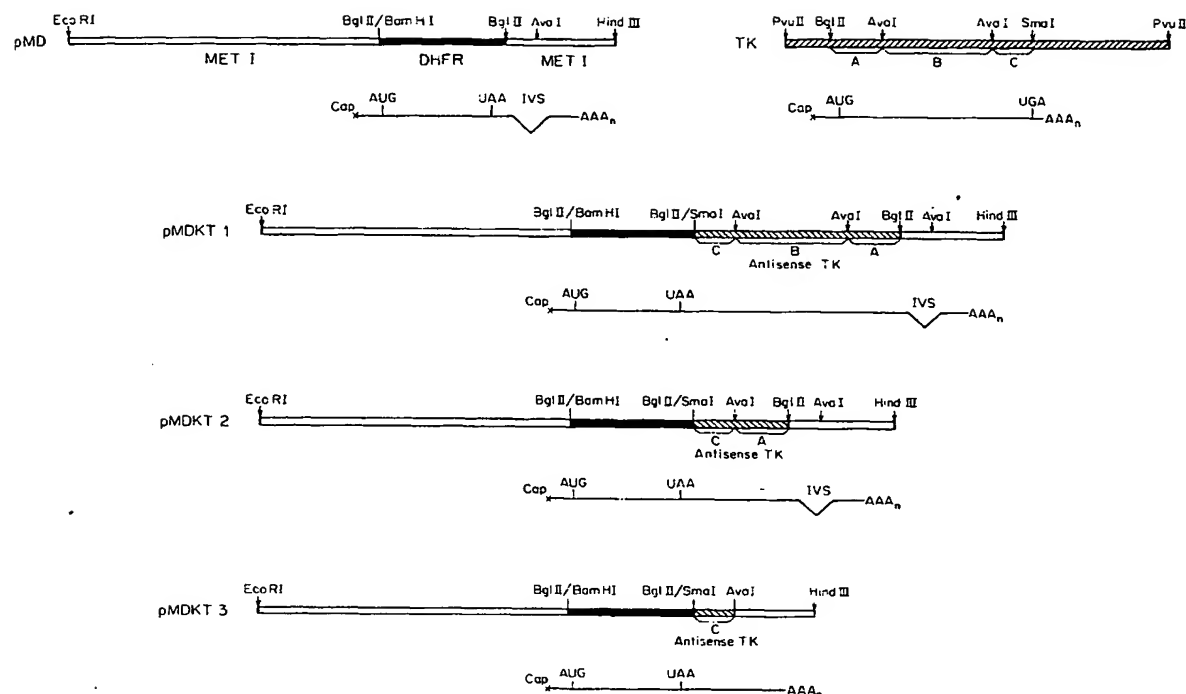


Figure 1. Anti-Sense Thymidine Kinase Genes and Their Derivation

Maps of eukaryotic DNA inserts of anti-sense genes pMDKT1, pMDKT2, pMDKT3, and the DNAs from which they were constructed, HSV thymidine kinase (McKnight, 1980) and pMD. Plasmid pMD contains 5' chromosomal sequences including the mouse metallothionein I promoter (Durnam et al., 1980; Glanville et al., 1981) joined to the mouse dihydrofolate reductase cDNA (Nunberg et al., 1980), followed by the intervening sequence and polyadenylation sequence of mouse metallothionein I. Selected restriction sites are indicated by arrows. Plasmid pMDKT1 contains a 1.1 kb fragment derived from the TK gene. This fragment, also used by Izant and Weintraub (1984), includes 53 bp encoding the 5' untranslated region of the TK mRNA and all of the protein coding sequence except the carboxy-terminal 23 bases. Plasmid pMDKT2 contains a 301 bp fragment from the 5' portion (segment A) and a 225 bp sequence from the 3' portion of the TK gene (segment C). Plasmid pMDKT3 contains only the terminal 225 bp fragment (segment C) corresponding to the carboxy terminus of thymidine kinase, and possesses a reduced 3' untranslated region from the metallothionein I gene. Junctions between two sites that were used in plasmid construction and which are no longer substrates for either parental enzyme are marked by a line perpendicular to the map. Translation initiation and termination codons are noted. See Experimental Procedures for details of construction. Each eukaryotic DNA insert is cloned in pBR322. Metallothionein sequence, open; DHFR sequence, solid; TK sequence, shaded; TK sequence in antisense orientation, shaded. Translational initiation and termination sites are indicated. Predicted primary transcripts are denoted by the line drawing below each DNA map.

cumulate significant quantities of anti-sense RNA. It is known that numerous chimeric genes containing the metallothionein promoter readily direct the synthesis of high levels of messenger RNA under induction conditions similar to those tested here (Mayo et al., 1982). A particularly relevant example is vector pMD (Figure 1), which contains the complete DHFR protein coding sequence in sense configuration flanked by promoter and 3' sequences from the metallothionein gene. Unlike the analogous gene containing DHFR sequences in the anti-sense orientation, pMD routinely produces substantial levels of mRNA upon heavy metal induction of cells containing the vector (P. Mueller and B. Wold, unpublished). Taken together, these results indicate that the failure to accumulate anti-sense transcripts may be due to posttranscriptional events such as aberrant processing, transport, or turnover.

Design of Amplifiable Anti-Sense RNA Genes

A second generation of plasmids producing higher steady

state levels of anti-sense RNA was designed. These genes contain the dihydrofolate reductase protein coding region (in the sense orientation) followed by anti-sense TK sequences (Figure 1). Their transcripts are expected to be bifunctional, directing the synthesis of an enzyme, DHFR, and providing anti-sense TK RNA. Dihydrofolate reductase levels can be significantly increased by selecting cells resistant to the drug methotrexate (Mtx). Methotrexate is a competitive inhibitor of DHFR and cells can become resistant to the drug by overproducing DHFR, usually by means of gene amplification (for a review, see Stark and Wahl, 1984). Since anti-sense TK sequences appear on the same RNA as DHFR sequences, selection for cells resistant to high levels of Mtx should result in the production of large amounts of anti-sense TK RNA.

In these experiments, three anti-sense genes were employed (Figure 1). The parental plasmid, pMD, contains a gene consisting of a metallothionein promoter, DHFR coding region, metallothionein intron, and metallothionein poly(A) addition site. Thymidine kinase DNA fragments

were inserted into the 3' untranslated region in inverted orientation relative to the DHFR sequences so that transcription originating from the metallothionein promoter would be expected to produce a messenger RNA coding for reductase and containing anti-sense TK sequences in its 3' untranslated region (details of anti-sense TK sequence content and construction appear in the legend to Figure 1 and in Experimental Procedures). The transcripts expected from each of these genes are indicated in Figure 1.

Low Levels of Anti-Sense TK RNA Do Not Affect Thymidine Kinase Activity

In order to construct a parental thymidine kinase positive cell line in which anti-sense TK genes would subsequently be tested, DNAs containing TK and adenine phosphoribosyl transferase (APRT) genes were introduced into L APRT⁺ TK⁻ cells by DNA mediated gene transfer (Wigler et al., 1977; see Experimental Procedures for details of those transfections). One APRT⁺ TK⁻ clone reverted to APRT⁺ TK⁻ at a frequency less than 10^{-5} revertants/cell in three independent experiments. This frequency of reversion is sufficiently low that thymidine kinase activity is expected to be unaffected by the accumulation of spontaneous TK revertants. Therefore, this clone, designated P, was used as the parental cell line into which anti-sense TK vectors were introduced. In all experiments that follow, P cells were grown in media that imposes selection for APRT.

Next, the pMD control gene and the anti-sense genes were introduced into the TK positive parental cell line using pNEO3 as the selectable cotransforming marker. The pNEO3 vector (B. Wold and R. Axel, unpublished results) expresses a bacterial enzyme that allows mammalian cells to grow in the presence of the drug G418 (Southern and Berg, 1982). Transformants from each plate were pooled. Five plates of parental cells were transfected with pMDKT1, resulting in five pools of transformants designated KT1-A to KT1-E. Similarly, cells transfected with pMDKT2 yielded KT2-A to KT2-E, cells transfected with pMDKT3 gave KT3-A to KT3-E, and cells transfected with pMD were designated KT0-A to KT0-C.

All of the transformants exhibited increased levels of resistance to methotrexate relative to parental cells, indicating that the newly transfected genes express dihydrofolate reductase. Prior to transfection, the parental cell line was resistant to 10 nM Mtx, whereas cells containing the DHFR chimeric genes were immediately resistant to elevated levels of methotrexate: KT1 cells were resistant to 50 nM Mtx, KT2 cells were resistant to 150 nM Mtx, and KT3 and KT0 cells were resistant to 300 nM Mtx. The variable levels of resistance to methotrexate indicate that differing amounts of DHFR are expressed in cells transfected with each gene.

Expression of anti-sense TK RNA in these primary transformant populations had no effect on the thymidine kinase phenotype. TK enzyme activity was quantitated in cells containing either the metallothionein-DHFR gene (pMD), or its anti-sense gene derivatives pMDKT1, pMDKT2, or pMDKT3 (Figure 1), respectively. The en-

Table 1. TK Activity is Diminished in Cell Lines that Are Resistant to Mtx

Cell Line	Vector	Mtx ^a	TK ^b	n ^c
P	NA	.01	1.0	(>4)
KT0	ApMD	.30	1.07	(2)
KT0-A-64	pMD	64	1.22	(2)
KT0-B	pMD	.30	1.13	(2)
KT0-B-64	pMD	64	.95	(2)
KT0-C	pMD	.30	1.05	(2)
KT0-C-64	pMD	64	.90	(1)
KT1-B-.05	pMDKT1	.05	1.0	(2)
KT1-B-2	pMDKT1	2	1.0	(4)
KT2-B-12	pMDKT2	12	.37	(2)
KT2-B-100	pMDKT2	100	.19	(>4)
KT2-D-9	pMDKT2	9	1.06	(>4)
KT2-D-100	pMDKT2	100	.17	(>4)
KT2-E-4	pMDKT2	4	.57	(>4)
KT2-E-24	pMDKT2	24	.52	(>4)
KT3-B-.15	pMDKT3	.15	1.03	(2)
KT3-B-64	pMDKT3	64	.15	(>4)
KT3-C-.15	pMDKT3	.15	.99	(2)
KT3-C-64	pMDKT3	64	.56	(3)
KT3-D-.15	pMDKT3	.15	1.04	(2)
KT3-D-64	pMDKT3	64	.18	(>4)
KT3-E-.15	pMDKT3	.15	1.07	(2)
KT3-E-64	pMDKT3	64	.80	(3)

^a Concentration of resistance to Mtx in μ M.

^b Relative amount of TK activity normalized to P cells. The TK activity in P cells was 1.58×10^{-3} U/mg protein.

^c Number of experiments.

zyme levels presented in Table 1, which are accurate to within 10%, show that the level of TK activity in these cells is identical with that expressed in the parental thymidine kinase positive cell line.

Selection for Methotrexate Resistance Results in Increased Levels of Anti-Sense TK RNA and Reduced TK Activity

In order to raise the concentration of the DHFR anti-sense TK RNA, cells resistant to higher levels of methotrexate were selected. Resistance of Mtx was gradually increased by successive rounds of selection in twofold greater drug concentrations (Alt et al., 1976). To establish that selection for methotrexate resistance resulted in elevated levels of anti-sense containing transcripts, RNA was extracted from KT3, KT3-B-0.3, KT3-B-2, and KT3-B-64 cells, which are resistant to 0.3 μ M Mtx, 2 μ M Mtx, and 64 μ M Mtx. These RNAs were analyzed for the presence of anti-sense TK RNA using an RNAase protection assay. Radioactively labeled TK RNA was synthesized in vitro using an SP6 transcription system (Melton et al., 1984). This probe was hybridized to cellular RNA, and unhybridized probe was digested with RNAases A and T1, which are specific for single-stranded RNA. The protected probe was melted and fractionated on a denaturing polyacrylamide gel. As seen in Figures 2A and 2B, approximately 300 times more anti-sense TK RNA is made in cells resistant to 64 μ M Mtx than in cells prior to selection for Mtx resistance. The subcellular distribution of anti-sense TK transcripts was also determined for KT3-B-64 cells. As shown in Figure 2C, about 60% of anti-sense TK RNA is present in the

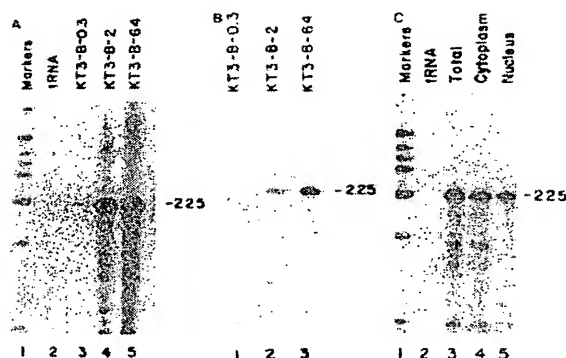


Figure 2. Anti-Sense Thymidine Kinase RNA Analyzed by RNAase Protection

(A) A uniformly radiolabeled 32 P sense strand RNA probe was synthesized in vitro by SP6 RNA polymerase. Excess probe was hybridized in solution with 2.5 μ g of total cellular RNA from each sample. Unhybridized RNA was digested by treatment with ribonucleases A and T1. Ribonuclease was removed and the protected RNA probe was melted and fractionated by electrophoresis through a 4% denaturing 8 M urea gel. The position expected for a 225 nucleotide fragment is indicated. Lane 1, DNA size markers; lane 2, 20 μ g yeast tRNA; lane 3, 2.5 μ g RNA from KT3-B-0.2 cells before selection for methotrexate resistance; lane 4, 2.5 μ g RNA from KT3-B-2 cells, resistant to an intermediate level of Mtx (2 μ M); lane 5, 2.5 μ g RNA from KT3-B-64 cells, resistant to a high level of Mtx (64 μ M); 25 hr exposure.

(B) Thirty minute exposure of the gel shown in (A).

(C) Subcellular distribution of anti-sense TK RNA in KT3-B-64 cells. RNA samples were analyzed by hybridization with uniformly radiolabeled sense TK RNA as described in (A). Lane 1, DNA size markers; lane 2, 20 μ g yeast tRNA; lane 3, 2.5 μ g KT3-B-64 total cellular RNA; lane 4, 2.5 μ g KT3-B-64 cytoplasmic RNA; lane 5, KT3-B-64 nuclear RNA extracted from the same cell lysate as was the cytoplasmic RNA of lane 4. The quantity of nuclear RNA hybridized is that which was extracted from the same number of cells as yielded 2.5 μ g of cytoplasmic RNA.

cytoplasm at steady state while the remaining 40% is nuclear. Similarly, in KT2-E cells, it was found that selection for Mtx resistance resulted in a large increase in the expression of anti-sense TK RNA (data not shown). Therefore, selection for resistance to methotrexate resulted in increased concentrations of DHFR anti-sense TK RNA in cells transfected with either pMDKT2 or pMDKT3. Examination of genomic DNA isolated from parental cells, initial transformants, and cells resistant to 64 μ M Mtx showed that the predominant means of generating higher levels of DHFR anti-sense TK RNA was by amplification of gene number (data not shown). However, this was not the case in cells transfected with pMDKT1 which contains the largest anti-sense TK sequence. In two independent cases, KT1 cells did not produce more anti-sense TK RNA in response to Mtx selection (data not shown). Because these cells showed no reduction in TK activity, and because there was no increase in anti-sense TK RNA upon methotrexate selection, they were not studied further.

Since stepwise selection for methotrexate resistance resulted in a gradual increase in the level of anti-sense TK RNA, it was anticipated that at some point the concentration of anti-sense RNA might become great enough to inhibit thymidine kinase expression. The level of TK enzyme

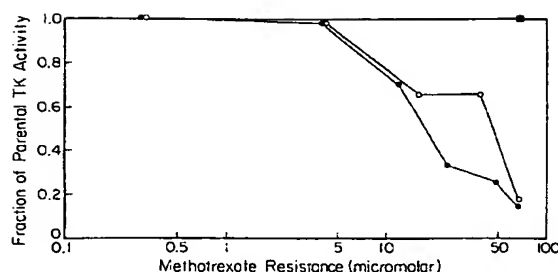


Figure 3. Thymidine Kinase Activity as a Function of Methotrexate Resistance

Thymidine kinase enzyme activity was determined as described in Experimental Procedures. TK activity is expressed as the fraction of enzyme activity expressed in parental (P) TK positive cells, and is plotted as a function of methotrexate resistance. KTO cells, containing the pMD gene that has no anti-sense TK sequences, solid boxes; KT3-B cells, containing anti-sense gene pMDKT3, solid circles; KT3-D cells, containing anti-sense gene pMDKT3, open circles.

Table 2. TK Activity in Cell Clones with Amplified Anti-Sense Genes

Cell Line	Vector	Type ^a	Mtx ^b	TK ^c	Hexokinase ^d
Parental	none	C	.01	1.00	1.00
KT3-B-64	pMDKT3	P	64	.15	
KT3-B.1-64	pMDKT3	C	64	.78	
KT3-B.2-64	pMDKT3	C	64	.44	
KT3-B.3-64	pMDKT3	C	64	.47	
KT3-B.5-64	pMDKT3	C	64	.11	1.06
KT3-B.6-64	pMDKT3	C	64	.16	1.03
KT3-B.7-64	pMDKT3	C	64	.18	.94
KT3-B.8-64	pMDKT3	C	64	.18	.84
KT3-B.9-64	pMDKT3	C	64	.35	

^a C, clone; P, population.

^b Concentration of resistance to Mtx in μ M.

^c Relative amount of TK activity normalized to P cells.

^d Relative amount of hexokinase activity normalized to P cells.

activity in KT3 cells was determined at each step in increased Mtx resistance. As can be seen in Figure 3, there is much less TK activity in KT3-B-64 or KT3-D-64 cells than there is in parental cells. Beginning at 4 μ M, the level of TK activity continually decreased until only 15% or 18% of the enzyme activity present in parental cells remained in KT3-B-64 and KT3-D-64 cells respectively. The level of TK activity for other transformants before and after Mtx selection is shown in Table 1. Two out of four independent pools of KT3 cells showed a significant decrease in TK activity in cells resistant to 64 μ M Mtx. Likewise, two out of three pools of KT2 cells exhibited reduced TK activity in cells resistant to 100 μ M Mtx.

Specificity of Anti-Sense RNA Effects

Control experiments showed that the reduction in TK activity in KT2 and KT3 cells apparently depends on the presence of anti-sense TK sequences. The plasmid pMD (Figure 1) is identical to the anti-sense genes in KT2 and KT3 cells except that it contains no anti-sense thymidine kinase sequences. When pMD containing cells had become resistant to 64 μ M methotrexate (the Mtx level at which KT2 and KT3 cells were showing TK depression),

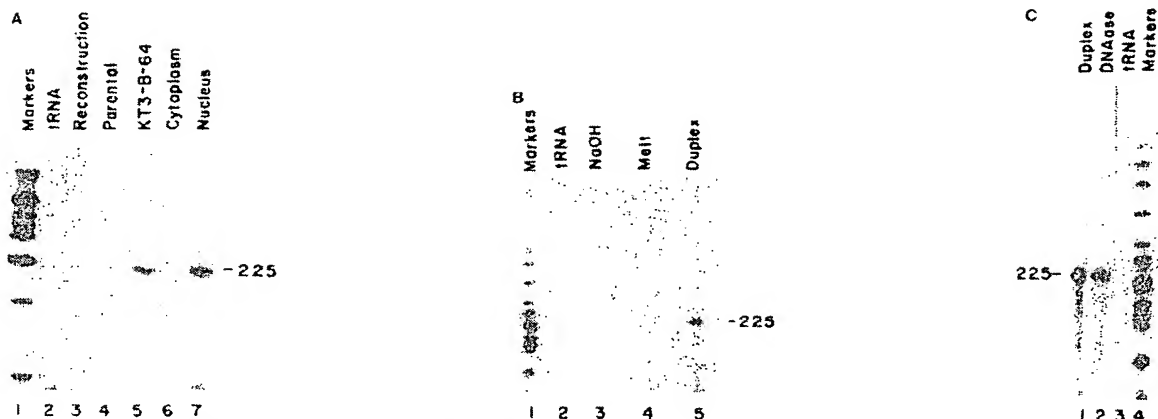


Figure 4. Detection of Double-Stranded Thymidine Kinase RNA

(A) RNA samples were digested with ribonucleases A and T1 under a condition that degrades single-stranded RNA but spares duplexes. Ribonuclease was eliminated, and surviving RNAase resistant material was combined with a uniformly radiolabeled ^{32}P RNA probe for sense TK RNA. The probe was prepared using SP6 polymerase and includes 1.3 kb of anti-sense TK sequence. The mixture was melted and allowed to reassociate in solution. Hybridization was terminated by a second ribonuclease digestion to eliminate unhybridized probe, and samples were melted and displayed on a denaturing 4% polyacrylamide gel. The expected position for a 225 nucleotide fragment is indicated. Lane 1, DNA size markers; lane 2, 25 μg of yeast tRNA; lane 3, 25 μg of total cellular RNA from the parental (P) cell line; lane 4, 25 μg of total cellular RNA from the reconstruction control (see text) in which in vitro synthesized anti-sense TK RNA was added to P cells at lysis and co-extracted; lane 5, 25 μg of total RNA from KT3-B-64 cells; lane 6, 25 μg of cytoplasmic RNA from KT3-B-64 cells; lane 7, nuclear RNA extracted from the same number of KT3-B-64 cells as yielded 25 μg of cytoplasmic RNA.

(B) Ribonuclease resistant duplexes from KT3-B-64 cells have properties expected for RNA:RNA hybrids. All RNA samples were analyzed for the presence of RNAase resistant TK duplexes as described in (A), after they had been subjected to initial treatments as indicated (see also Experimental Procedures). Lane 1, DNA size markers; lane 2, 25 μg yeast tRNA; lane 3, 25 μg of total RNA from KT3-B-64 cells subjected to base hydrolysis and neutralization prior to analysis; lane 4, 25 μg of total KT3-B-64 RNA melted prior to RNAase digestion; lane 5, 25 μg of total KT3-B-64 RNA without prior treatment.

(C) Duplexes from KT3-B-64 RNA are unaffected by stringent DNAase digestion. RNA samples were analyzed for the presence of RNAase resistant TK duplexes as described in (A), after initial treatment as indicated. Lane 1, 20 μg KT3-B-64 total cellular RNA; lane 2, 20 μg KT3-B-64 total cellular RNA treated with 250 μg per ml deoxyribonuclease I prior to analysis; lane 3, 20 μg yeast tRNA; lane 4, DNA size markers.

they were assayed for TK activity (Table 1 and Figure 3). Three independent populations of these cells, designated KTO-64, exhibited no detectable reduction in enzyme activity, indicating that the reduced TK phenotype observed in several KT2 and KT3 populations requires anti-sense TK sequence and is not merely an artifact of methotrexate selection or culture conditions.

A second set of control experiments showed that the reduction in thymidine kinase activity in KT2 and KT3 cells is specific for TK, whereas an arbitrarily selected enzyme activity was unaltered. The level of hexokinase activity is essentially unchanged in cell clones that have as much as a tenfold reduction in TK activity (Table 2).

Double-Stranded RNA Is Found in KT3-B-64 Nuclei

The results presented so far suggest that TK activity can be specifically reduced in cells that express high concentrations of anti-sense TK RNA. We have examined one cell population, KT3-B-64, in greater detail for the presence of double-stranded RNA. RNA samples were treated with RNAases A and T1, which digest single-stranded RNA, but leave double-stranded RNA intact. The ribonucleases were removed and the RNAases resistant fraction was denatured and hybridized to an RNA probe for sense TK RNA. After hybridization, unreacted probe was digested by RNAase A and T1, then fractionated on a denaturing

polyacrylamide gel. As expected, the TK positive parental cell line (P) and TK positive cells expressing low levels of anti-sense RNA (KT3-B-0.3) contain no detectable double-stranded TK RNA (Figure 4A, lane 4, and data not shown). However, a band was observed when RNA samples from the TK diminished population, KT3-B-64, were analyzed for TK RNA hybrid content (lane 5).

Several control experiments show that the protected band in Figure 4A is due to the presence of RNA:RNA hybrids in KT3-B-64 RNA rather than containing genomic DNA. It is expected that bonafide RNA:RNA duplexes will be labile to ribonuclease digestion if they are first heat denatured, and that they will be destroyed by alkaline hydrolysis. The 225 nucleotide signal observed in KT3-B-64 RNA is sensitive to both of these pretreatments as shown in Figure 4B. Furthermore, RNA hybrids should be resistant to exhaustive DNAase digestion, whereas contaminating DNA would be sensitive. As shown in Figure 4C, the band at 225 nucleotides is qualitatively and quantitatively unaffected by stringent prior DNAase treatment. We conclude that the signal observed is due to RNA duplexes in KT3-B-64 RNA.

Results of another experiment presented in Figure 4A, lane 3, argue that the RNA duplexes detected in KT3-B-64 RNA are formed in vivo and are not a product of reassociation of sense and anti-sense RNAs during RNA extraction

and handling. No double-stranded RNA was observed when anti-sense TK RNA was synthesized *in vitro* and added to parental TK positive cells at the start of RNA preparation. The amount of anti-sense TK RNA that was added to P cells was equivalent to the amount of anti-sense TK RNA in KT3-B-64 cells (50 ng anti-sense TK per 100 μ g of P cell total RNA). This experiment suggests that the double-stranded TK RNA in KT3-B-64 cells did not form during RNA preparation and that it is probably present *in vivo*, although it is not possible to formally exclude that hybridization occurs in the cell at the instant of lysis. The size of the double-stranded RNA, 225 bp, is the same as the size of anti-sense TK sequences contained in the DHFR anti-sense TK gene of KT3 cells (pMDKT3), suggesting that the entire anti-sense TK RNA sequence is present in the double-stranded RNA.

Hybridization of anti-sense TK RNA with sense TK RNA might occur in the cytoplasm and reduce thymidine kinase levels by inhibiting translation of TK mRNA, or it might occur in the nucleus resulting in inhibition of RNA processing or transport. In order to distinguish between these possibilities, KT3-B-64 cells were fractionated and nuclear and cytoplasmic RNAs were prepared. The amount of double-stranded TK RNA was measured using an RNAase protection assay as outlined above. The striking result is shown in Figure 4A, lanes 6 and 7. At least 95% of the double-stranded TK RNA is confined to the nuclear fraction. In this experiment each hybridization sample contains RNA extracted from the same number of cells. The RNA samples have not been subjected to oligo(dT)-cellulose fractionation, so there is no selection based on polyadenylation. Comparison of the amount of hybrid detected in total cellular and nuclear RNA samples from equivalent numbers of cells suggests that all of the hybrid in the total sample can be accounted for in the nuclear fraction.

Sense TK RNA Accumulates in the Nucleus of KT3-B-64 Cells

Sense TK RNA from parental cells and KT3-B-64 cells was analyzed. RNA samples were melted and hybridized with an RNA probe for sense TK RNA. Unhybridized probe was digested with RNAase A and T1, and the protected probe was run on a nondenaturing polyacrylamide gel. Since the RNA sample was melted before hybridization, this procedure should detect all sense TK transcripts regardless of whether they are single-stranded or double-stranded in the cell. As can be seen in Figure 5A, KT3-B-64 cells show similar, if not increased, levels of sense TK RNA when compared to parental cells. However, KT3-B-64 cells have eight times less TK activity, suggesting that the sense TK RNA is inefficiently utilized. In order to explore the basis of this poor expression, the abundance of the sense TK RNA in the nuclear and cytoplasmic fractions was analyzed. It was found that in KT3-B-64 cells greater than 95% of the sense TK RNA is localized in the nucleus, whereas in parental cells the sense TK RNA is primarily cytoplasmic (Figure 5B and data not shown). In this experiment, as before, each hybridization contains RNA from an equal number of cells. This result suggests

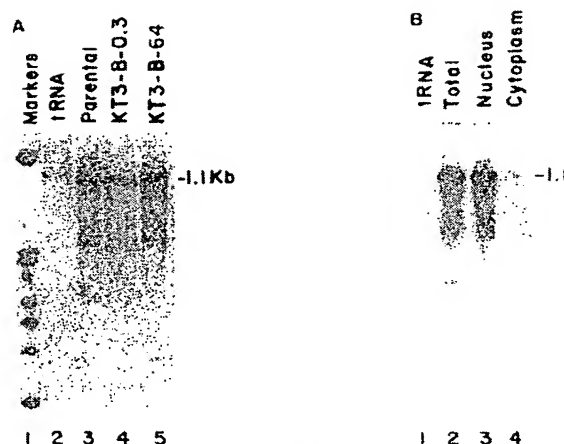


Figure 5. Sense Strand Thymidine Kinase RNA Analyzed by RNAase Protection

(A) RNA samples were hybridized with uniformly radiolabeled 32 P anti-sense TK RNA probe to detect the presence of sense transcripts. RNA samples were combined with 2×10^6 cpm of probe, melted, and allowed to anneal in solution (see Experimental Procedures). Ribonuclease resistant products are displayed on a neutral 4% polyacrylamide gel. The electrophoretic origin is at the top, and the expected migration distance for a 1.1 kb protected duplex is indicated. Lane 1, DNA size markers; lane 2, 20 μ g yeast tRNA; lane 3, 12 μ g total RNA from parental P cells; lane 4, 12 μ g of total RNA from KT3-B-2 cells; lane 5, 12 μ g of total RNA from KT3-B-64 cells.

(B) RNA samples were analyzed as in (A). Lane 1, 20 μ g yeast tRNA; lane 2, 12 μ g KT3-B-64 total cellular RNA; lane 3, KT3-B-64 nuclear RNA derived from a cell number identical to that which yielded 12 μ g of cytoplasmic RNA; lane 4, 12 μ g of cytoplasmic KT3-B-64 RNA.

that the decreased thymidine kinase activity is caused by a lack of sense TK messenger RNA in the cytoplasm where it can be translated.

Only 225 bp of TK sequence are in hybrid form in the nuclear RNA of KT3-B-64 cells, because there are only 225 bases of anti-sense sequence in this particular anti-sense gene. However, when the nuclear sense TK RNA is examined, the majority is at least 1.1 kilobases in length (this corresponds to the full TK sequence assayed by the RNA probe, and constitutes 90% of a full-length TK transcript). Therefore, once hybridized, the free ends of the single-stranded sense TK RNA appear not to be degraded much more rapidly than the double-stranded core. Quantitation of sense TK and duplex TK RNA was performed by excising the relevant bands from gels like those shown in Figures 4 and 5, and counting the samples. Given the specific activity of the SP6 RNA probe, it was calculated that there are about 50 molecules of duplex thymidine kinase RNA per cell. Within the limits of the measurement, this accounts for all of the sense TK transcript in KT3-B-64 nuclei, and implies that more than half of this RNA is in hybrid form.

Discussion

The results presented here suggest that high levels of cellular anti-sense TK RNA can stably reduce thymidine kin-

ase activity. Genes capable of producing DHFR anti-sense TK RNA were introduced by DNA transfer into TK⁺ mouse L cells. Resistance to high concentrations of Mtx usually involves overexpression of DHFR RNA which, in this experiment, also results in an increase in the amount of linked anti-sense TK RNA. Selection for Mtx resistance greatly increased the concentration of DHFR antisense TK RNA. The predominant mechanism of increased expression was amplification of the copy number of the DHFR antisense TK genes. In several independent cell populations, the TK level was diminished when the concentration of anti-sense TK RNA became high. Double-stranded TK RNA was observed in KT3-B-64 cells, which have decreased TK activity, but was not found in KT3-B-0.3 cells or parental cells, which have full TK activity. The double-stranded RNA was predominantly nuclear rather than cytoplasmic, and sense TK RNA was also localized to the nucleus.

The Mechanism of TK Reduction by Anti-Sense RNA

Based on the observations presented above, we prefer the following mechanism for the diminution of TK activity in KT3-B-64 cells. When the concentration of anti-sense TK RNA becomes sufficiently high, it hybridizes with sense TK RNA in the nucleus, and the double-stranded RNA is poorly transported to the cytoplasm. As a result, the level of TK enzymatic activity diminishes because relatively less sense TK RNA appears in the cytoplasm where it can be translated. However, it is not possible to draw conclusions regarding kinetic processes based solely on steady state measurements such as those presented here, and there are other models that are consistent with the data. For example, the failure to detect cytoplasmic double-stranded RNA could be explained by cytoplasmic hybridization followed either by transport of the double-stranded RNA to the nucleus or by its rapid degradation in the cytoplasm.

Anti-Sense RNA Physiology

The level of accumulation of anti-sense TK RNA differed depending on the type and amount of anti-sense TK sequence present in the chimeric gene. Several observations made in the course of this study suggest that in the pMDKT1 gene, inclusion of a 1.1 kb fragment of anti-sense TK sequence interfered with the accumulation of transcripts from this gene. First, the initial resistance to methotrexate following DNA mediated gene transfer with pMDKT1 is six times lower than the resistance obtained with pMD (an identical gene in all respects except that it contains no TK sequence). Second, selection of Mtx resistance was much more difficult in cells containing this gene than in cells containing DHFR genes with smaller segments of anti-sense TK sequence (pMDKT2 and pMDKT3) or no TK sequence at all (pMD). Finally, when Mtx resistant KT1 cells were obtained, the anti-sense TK sequences were not expressed at a higher level but appeared as a smear on a Northern blot (data not shown). In contrast, pMDKT2 and pMDKT3 do not show this severe interference with DHFR expression. These genes dif-

fer from pMDKT1 in that they lack a central Ava I fragment of the anti-sense TK gene and produce an RNA that is smaller. The reasons for the difference in DHFR expression between these genes are incompletely understood. A practical implication is that in future experiments with other genes it may be necessary to identify and remove anti-sense sequences that interfere with the accumulation of anti-sense RNA on a case by case basis.

In the experiment described here, thymidine kinase activity was significantly diminished only when a high level of anti-sense TK RNA was expressed. In KT3-B-64 cells, a steady state level of at least $5-10 \times 10^3$ anti-sense TK RNA molecules per cell is maintained, with about 40% localized in the nuclear compartment and 60% in the cytoplasm (Figure 2). Thus, in KT3-B-64 cells, there is at least a 300-fold excess of anti-sense TK RNA over sense TK RNA, as well as a high absolute intracellular concentration. In particular, the high nuclear concentration is probably responsible for the phenotypic effects.

The levels of anti-sense RNA reported here appear to be significantly higher than the amounts reported in the experiments of Izant and Weintraub (1984). In that study, the quantities of cytoplasmic anti-sense TK RNA and sense TK mRNA were approximately equal. The level of nuclear anti-sense RNA was not determined, and if it were high, it might have contributed significantly to the observed effect on TK activity. The origins of this difference in effective RNA levels may also result from any of the several differences in experimental design, including stable versus transient inhibition, translated versus untranslated anti-sense RNAs, and newly microinjected versus stably integrated TK genes.

As shown in Table 2, the level of TK activity varied in different cell clones derived from the KT3-B-64 cell population. The level of TK activity in the clones ranged from 80% in clone KT3-B.1-64 to 11% in clone KT3-B.5-64. One explanation for the difference in TK activity is that the levels of anti-sense TK RNA may fluctuate between these clones. Another possibility is that different clones vary in the location of sense and anti-sense sequences in the genome, and the inhibition of TK expression may be more efficient when the anti-sense genes are close to the sense gene creating high local concentrations of anti-sense RNA.

At present, we have only limited data on effects generated by different regions of anti-sense TK sequence. In two previous studies, one in *E. coli* and the other in *Xenopus* oocytes, it was found that anti-sense RNA was effective only if sequences complementary to the AUG start codon were included (Coleman et al., 1984; Melton, 1985). This supports the idea that in these systems the principal effect of anti-sense RNA is exerted at the translational level. Perhaps this is not surprising since in the *Xenopus* experiments both sense and anti-sense RNAs were injected directly into the cytoplasm, precluding any effects at earlier stages in RNA biogenesis; and in *E. coli*, transcription and translation are tightly coupled in time and location leaving few intermediate events to be blocked. In the animal cell experiments reported here we have found that anti-sense sequences derived exclusively from the 3'

region of TK protein coding sequence are sufficient to exert a phenotypic effect. Furthermore, a second anti-sense gene (pMDKT2), which contains sequences spanning the initiation AUG in addition to the carboxy-terminal sequence of pMDKT3, was neither more nor less effective in diminishing TK activity than was the carboxy segment acting alone.

The results presented here, taken together with recent studies in amphibian oocytes (Melton, 1985), *Drosophila* embryos (Rosenberg et al., 1985), and mammalian cells in culture (Izant and Weintraub, 1984) indicate that experimentally induced anti-sense RNAs can exert profound effects on the expression of their corresponding gene products. Although the mechanisms by which these effects are mediated might differ from case to case, the most important fact may be that such effects occur at all. This raises the interesting question of whether in eukaryotes complementary RNAs other than the experimentally induced species studied thus far are permitted to hybridize in vivo. There is a wealth of data showing that complementary RNA species are present at substantial concentrations both in nuclei (Federoff et al., 1977; Scheller et al., 1978; Davidson and Posakony, 1982) and in cytoplasm (Costantini et al., 1980; Anderson et al., 1982; Jelenik et al., 1978), but it is not known whether these transcripts exist in duplex form in vivo. Considered together with the fact that there are now several well documented instances in which RNA hybridizations play a specific regulatory role in prokaryotes (Simons and Kleckner, 1983; Mizuno et al., 1984; Tomizawa, 1984), it seems plausible that eukaryotes may also mediate some regulatory events through in vivo hybridization.

Application of Anti-Sense RNA Analysis

The data presented in this report argue strongly that anti-sense TK RNA can significantly diminish thymidine kinase activity. It remains possible that the TK gene is a special case and, in general, anti-sense RNA will be ineffective in inhibiting the expression of other genes. Salient differences between the Herpes virus thymidine kinase gene and other genes include the fact that the TK gene was introduced into the genome by DNA transfer, the gene is relatively small (1.3 kb), and it contains no intervening sequences. However, it seems unlikely that any of these characteristics would make HSV TK an especially favorable target for anti-sense RNA effects. In fact, these features may actually make TK a particularly difficult gene for anti-sense analysis. For example, the 3' end of TK mRNA, which was hybridized with anti-sense RNA in these experiments, is the last sequence to be transcribed and must therefore have a somewhat shorter nuclear dwell time than upstream sequences. In addition, the lack of an RNA splicing step may permit more rapid nuclear transport for this RNA than for typical spliced RNAs. This line of reasoning suggests that RNA transcribed from the 5' region of large genes containing multiple intervening sequences will likely take longer to be exported from the nucleus than would 3' sequences from small genes such as TK. Therefore, anti-sense RNA complementary to 5' sequences from long chromosomal genes may be most sus-

ceptible to nuclear hybridization with complementary RNA.

In this study, an amplification protocol was useful because it permitted us to test the effects of a wide range of anti-sense levels. This was important since we had no knowledge, *a priori*, of the quantity of anti-sense transcript that would be required to have a significant impact on cellular phenotype. Based on the results of this experiment, it seems possible that in future studies effective levels of anti-sense RNA might be established more quickly by the use of multicopy episomal vectors such as those derived from bovine Papilloma virus (Law et al., 1983). A second desirable modification of the approach tested here could be provided by strongly conditional expression of anti-sense RNA. In KT3-B-64 cells the level of anti-sense RNA transcribed from the metallothionein promoter is only slightly increased when the cells are treated with heavy metals (P. Mueller and B. Wold, unpublished observations). Therefore, the original goal of partially conditional expression of DHFR antisense TK RNA in this experiment was not met in practice. This indicates that alternative regulated promoters should be used in future experiments.

Another issue regarding the utility of anti-sense RNA is whether a reduction of tenfold in gene activity is sufficient to cause a hypomorphic phenotype. A tenfold reduction of TK activity did not render these cells resistant to bromodeoxyuridine and therefore TK⁻, as conventionally defined (unpublished observations). Thus, this technique is not yet useful for establishing the complete loss of function of a gene. However, there are a number of genes that show phenotypic effects due to only a partial loss of function. In genes that exhibit haplo-insufficiency, such as the *Notch* locus in *Drosophila*, a twofold difference in gene product yields an altered phenotype (Wright, 1970). Furthermore, there are cases in which the hypomorphic phenotype may reveal subtleties about the function of the gene product that are not obvious from the null phenotype. For example, a 90% or greater deficit in human low density lipoprotein receptor grossly affects cholesterol metabolism and is often fatal in childhood (Goldstein and Brown, 1983). However, a twofold reduction in receptor level makes the affected individual prone to premature atherosclerosis and cardiovascular disease. These examples suggest that there are likely to be other genes that will exhibit a change in cellular phenotype when their product level is diminished by two to tenfold. In addition, any future improvements that extend the level of phenotypic reduction beyond a factor of ten would be expected to increase the range of genes for which anti-sense RNA analysis could be fruitful.

Experimental Procedures

Plasmid Construction

Plasmid pMD, a gift from P. Mueller, was constructed from the metallothionein I gene and the DHFR cDNA (Durnam et al., 1980; Southern and Berg, 1982). A Bam HI to Bgl II fragment containing the DHFR cDNA was inserted into the metallothionein I gene, which had been previously cleaved by Bgl II, located 9 bp upstream of the ATG start codon, and Alu I, located in the second exon. The Alu I site in the metallothionein gene was converted into a Bgl II site by the addition of Bgl

II linkers. Plasmid pMDKT1 was made by inserting a 1.1 kb Bgl II to Sma I fragment derived from the HSV TK gene into the Bgl II site in pMD after the attachment of a Bam HI linker to the Sma I site. Plasmid pMDKT2 was made by partially digesting pMDKT1 with Ava I and isolating a plasmid that had the 600 bp Ava I fragment from the middle of the TK sequences deleted. Plasmid pMDKT3 was made by deleting a 1050bp Ava I fragment from pMDKT1 and recircularizing.

Cell Culture

L cells were maintained in Dulbecco's modification of Eagle's Medium supplemented with 10% calf serum, 10 µg/ml streptomycin sulfate, and 60 µg/ml penicillin-K (EC₁₀ media). APRT selection used EC₁₀ media supplemented with 4 µg/ml azaserine and 15 µg/ml adenine. P cells and all cells derived from them were grown in EC₁₀ supplemented with azaserine, adenine, and 1x nonessential amino acids (EC₁₀AAN media). Selection for cells resistant to Mtx was accomplished by the procedure of All et al. (1976). Approximately 2 to 5 × 10⁵ cells in a 100 mm plate were placed in EC₁₀AAN medium, which contained a twofold higher concentration of Mtx. After about 2 weeks, the plates contained approximately 10⁴ cells and the process was repeated after splitting the cells appropriately. Since thymidine, the substrate used by TK, is not added to EC₁₀AAN media, the absence or presence of TK activity should not affect the growth of cells under Mtx selective pressure. Cells were transformed by the procedure of Wigler et al. (1977).

DNA Transformation and Construction of Cell Lines

To construct the parental thymidine kinase positive cell line in which anti-sense TK genes would subsequently be tested, DNAs containing TK and adenine phosphoribosyl transferase (APRT) genes were introduced into L APRT⁻ TK⁻ cells by DNA mediated gene transfer (Wigler et al., 1977).

To construct anti-sense cell lines, each 100 mm plate of 5 × 10⁵ parental cells received 1 µg pNEO3 DNA, 10 µg carrier DNA, and 10 µg of mMD, pMDKT1, mMDKT2, or pMDKT3 DNA as a calcium phosphate precipitate. The pNEO3 vector (B. Wold and R. Axel, unpublished results) expresses a bacterial enzyme that allows mammalian cells to grow in the presence of the drug G418 (Southern and Berg, 1982).

Enzyme Assays

TK activity was assayed by a modification of the procedure of Wigler (1977). The cells from one 100 mm plate near confluence were washed with cold PBS, scraped in 5 ml of cold PBS, and pelleted. The pellet was resuspended in 150 µl of cold TK lysis buffer (10 mM Tris-HCl, pH 7.8, 10 mM KCl, 1 mM MgCl₂, 1 mM β-mercaptoethanol, and 20 µM thymidine). The cell suspension was frozen and thawed four times in dry ice. To the solution was added 25 µl of 1.0 M KCl, and the cells were spun for 10 min in an eppendorf microfuge at 4°C on ice; 25 µl of reaction buffer (150 mM Tris-HCl, pH 7.8, 20 mM MgCl₂, 25 mM KCl, 1.5 mM β-mercaptoethanol, 60 mM ATP, and 75 µCi/ml [³H]-thymidine, 50 Ci/mole) was added to 50 µl of cellular supernatant and mixed. Each sample was done in duplicate at several different lysate concentrations. As a negative control, lysis buffer plus KCl was used; neither BSA nor an extract made from L APRT⁻ TK⁻ cells gave a signal above the background value using lysis buffer alone. The rack containing the samples was placed in a 37°C water bath for 30 min. The rack was placed on ice and the reactions were terminated by spotting 50 µl on DE81 disks. The disks were washed twice in 1 mM ammonium formate, once in water, and once in ethanol for 5 min each with shaking at room temperature. The filters were dried, added to 10 ml hydrofluor, and counted in a scintillation counter. Under these conditions, the reactions are linear both for amount of cell extract, from 5 µg to 150 µg total cell protein, and for time, up to 90 min. The four samples usually gave values to within 10% of each other. The ATP concentration in the reaction mix was increased from 15 mM to 60 mM in order to keep the reaction linear for 90 min. Protein content was measured by the method of Bradford (1976). One unit is defined as that amount of enzyme required to convert 1 nmole thymidine into TMP in 1 min. Hexokinase activity was measured as previously described (Walker and Parry, 1966).

Synthesis of RNA Probes with SP6 Polymerase

A 1.1 kb Bgl II to Sma I fragment from the HSV TK gene was inserted into the Bgl II site of pSP6-4T such that sense TK sequences were tran-

scribed and was therefore used as a probe for anti-sense TK RNA. A 1.3 kb Eco RI to Sma I fragment from the HSV TK gene was inserted between the Eco RI and the Sma I sites of pSP6-4T and was used as a probe for anti-sense RNA. Both vectors were linearized at their unique Eco RI site prior to transcription. Synthesis of RNA probes by SP6 polymerase was done essentially as described (Melton et al., 1984). The transcription reaction did not contain bovine serum albumin. The RNA was labeled with 100 µCi of ³²P-GTP (Amersham, 400 Ci/nmole) diluted with unlabeled GTP to a final concentration of 40 µM. Other nucleotides were present at 500 µM. RNA was separated from unincorporated nucleotides by sepharose G50 chromatography and DNA was removed by selecting for poly(A)⁺ RNA on an oligo(dT) column (pSP6-4T contains poly(A) sequences), or by treatment with RNAase-free DNAase at 20 µl/ml, 37°C for 30 min.

RNAase Protection

Total cellular RNA was prepared by the method of Chirgwin et al. (1979) except that the DNA in the initial lysate was sheared by sonication. Nuclear and cytoplasmic RNA was prepared by the method of Favalaro et al. (1980). Typically, 3 × 10⁶ cells were lysed in an initial volume of 10 ml and yielded 1 mg of total RNA. RNA was prepared quickly and was never left unfrozen longer than necessary.

RNAase protection experiments were performed as described (Zinn et al., 1983). To a sample of RNA to be tested (1 µg to 50 µg) was added 2 × 10⁶ cpm of labeled probe (1 × 10⁶ cpm/µg), and the sample was precipitated in 2 M ammonium acetate and 1 volume isopropyl alcohol. In the experiments described in Figure 5, in which sense TK RNA was assayed in the presence of large amounts of anti-sense TK RNA, 5 × 10⁶ cpm of probe was used in order to insure a mass excess of probe. The dried RNA was dissolved in 30 µl of RNA hybridization buffer (80% formamide, 40 mM Pipes, pH 6.4, 400 mM NaCl, 1 mM EDTA), melted at 85°C for 10 min, and hybridized at 45°C overnight. A 300 µl addition of RNAase buffer (10 mM Tris-HCl, pH 7.8, 5 mM EDTA, 300 mM NaCl) containing 40 µg/ml RNAase A and 2 µg/ml RNAase T1 degraded unhybridized probe after incubation at 30°C for 30 min. Control experiments showed that essentially all single-stranded probe was digested using one-tenth of the above RNAase concentration, whereas the double-stranded RNA was resistant to 30 times the above RNAase concentration. The RNAases were inactivated by adding 10 µl of 20% SDS and 50 µg of proteinase K incubating at 37°C for 15 min. The RNA was extracted with phenol and then precipitated three times from ammonium acetate as before after the addition of 25 µg of carrier tRNA. When the protected probe size was expected to be under 1 kb in length, the samples were dissolved in loading buffer (80% formamide, 40 mM Tris-borate, pH 7.5), melted at 85°C for 10 min, and loaded onto 4% denaturing polyacrylamide gels (Maxam and Gilbert, 1980). Single-stranded fragments were more susceptible to degradation by radiolysis than double-stranded fragments. Therefore, for fragments greater than 1 kb in length, neutral polyacrylamide gels, using TAE buffer (50 mM Tris-acetate, pH 7.5, 1 mM EDTA), were used to analyze the undenatured RNA:RNA duplex.

In the experiments of Figures 4B and 4C, total KT3-B-64 RNA was treated initially by heat denaturation, base hydrolysis, or DNAase I digestion. Heat denaturation was performed in hybridization buffer (80% formamide, 0.4 M NaCl, 40 mM Pipes, pH 6.4, 1 mM EDTA) at 100°C for 4 min. The sample was quenched in a dry ice ethanol bath and diluted into 300 µl RNAase buffer as described above. Ribonuclease digestion, removal, and subsequent hybridization with SP6 probes was performed as described. Base hydrolysis pretreatment was performed by incubating RNA in 0.3M NaOH at 37°C for 30 min. Base treatment was terminated by the addition of 100 mM Tris, pH 7.5, 10 mM EDTA to 200 µl, and neutralizing at 4°C with 50 mM HCl. Thirty micrograms of carrier tRNA were added and the mixture was precipitated from isopropanol. The sample was resuspended, combined with radiolabeled probe for TK sequences, and hybridized and analyzed as described above. DNAase pretreatment was performed by incubating the RNA sample in DNAase buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂) and 250 µg per ml RNAase-free DNAase I (Worthington) for 1 hr at 37°C. The sample was diluted to 250 µl in proteinase K buffer, digested for 15 min, extracted once with phenol chloroform (1:1) and once with chloroform, and precipitated by the addition of 1 volume of isopropanol. The RNA was then analyzed for the presence of TK RNA hybrids as described above.

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Note Added in Proof

Recently Izant and Weintraub have also found that cells stably transformed with anti-sense HSV TK genes express diminished levels of TK activity. They also find that anti-sense TK RNA expressed from the mouse mammary tumor virus promoter can provide for conditional reduction in TK activity (Science, in press).